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INDIRECT HAEMAGGLUTINATION REACTION WITH ANTIGEN OF SARCOCYSTIS GIGANTEA (RAILLIET, 1886) ASHFORD, 1977

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Abstract. The water extract from cryolyzed whole muscle cysts of *Sarcocystis gigantea* from sheep, in spite of the high lectin content, is a suitable antigen for the detection of specific antibodies by means of indirect haemagglutination reaction (IHA). The agglutinating effect of lectin from parasitic cysts can be eliminated with a 0.5 % concentration of lactose dissolved in all solutions used for IHA. In sera of slaughterhouse sheep, positive titres ranging from 1 : 80 to 1 : 1 280 were registered. Positive reactions in lower titres were observed also with antibodies against *S. dispersa*, *S. cuniculi* and *Sarcocystis* sp. from pigs. Sensibilized erythrocytes can be stored in refrigerator at least for 1 week.

After successful experiments with the indirect haemagglutination reaction (IHA) in the detection of serum antibodies in mice experimentally infected with *Sarcocystis dispersa* (Červa and Černá 1980, 1982), attempts were made to use this method for the detection of antibodies against *S. gigantea* in spontaneously infected sheep. This reaction has already been used for the diagnosis of sarcosporidiosis in cattle and sheep, using the antigen prepared from cystozoites obtained from heart muscle of calves experimentally infected with *S. cruzi* (Lundė and Fayer 1977, Frelier et al. 1977, Leguia and Herbert 1979). This paper presents the results of application of IHA with antigen prepared from cystozoites of *S. gigantea* and necessary modifications of this method resulting from the presence of lectin in this antigen.

MATERIAL AND METHODS

Antigens used. One of the aims of the present study was to prepare from *S. gigantea* cysts an antigen applicable for the IHA reaction and to test its effect in the detection of homologous antibodies in sheep and heterologous antibodies in animal species infected with other species of *Sarcocystis*. The antigen of *S. dispersa* used in comparative experiments was prepared as described in a previous paper (Červa and Černá 1982).

Preparation of *Sarcocystis gigantea* antigen. Sheep oesophagi and diaphragms with *S. gigantea* cysts were obtained from animals slaughtered at the abattoir. By means of eye scissors and pincette the muscle tissue surrounding individual sarcocysts was carefully cut and whole undamaged cysts were pressed out and transferred to PBS (NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 . 12 H_2O 3.21 g, KH_2PO_4 0.2 g, H_2O dist. ad 1 000 ml). The cysts washed in PBS were gradually crushed in a glass piston homogenizer, always 40 cysts in 3 ml of PBS. The released cystozoites were separated from the remnants of cyst walls by 5 × repeated free sedimentation of the homogenate diluted with PBS excess. The supernatant of cystozoites gathered and their suspension was then washed 5 × by centrifugation in PBS (1 500 r.p.m. for 10 min, r = 14 cm). The sediment was transferred to distilled water and cryolyzed (= antigen I in Table 1). The sediment containing remnants of cyst walls was transferred to distilled water and cryolyzed (= antigen II). White opalescing PBS used for washing of corpuscular components of the homogenate was collected and also used as antigen (= antigen III). The quantitative data are summarized in Table 1. The antigens marked I and II were cryolyzed 5 × and centrifuged and only supernatant was used for all further determinations.

Sera tested. Sera of three groups of sheep were used in the experiments verifying the possibility of detection of homologous antibodies. The first group of sera was obtained from sheep of a herd with high spontaneous *S. gigantea* infection. The sera were collected from animals slaughtered at

the abattoir. In addition to macroscopical cysts of *S. gigantea*, the muscles of oesophagi of all sheep examined contained microscopical cysts corresponding in their morphology to the species *S. tenella* (*S. ovicanis*) after Levine and Tadros (1980). The second set of sera was collected from 20 lambs of the same herd at the age of 10 weeks. The third group of sera originated from 50 sheep bought by the abattoir from various regions of Bohemia. None of the sheep contained macroscopical cysts of *S. gigantea* in muscles of oesophagus and diaphragm, but all of them harboured the microscopical type of muscle cysts of *Sarcocystis*.

Table 1. Quantitative data on materials used for the preparation of *Sarcocystis gigantea* antigen

Material	Volume in ml	Wet weight in g	Protein nitrogen (Lowry)	Note
120 muscle cysts	6.8	5.3	—	
I = washed zoites	—	0.5	10.3 mg/ml	cryolyzed in 5 ml of H_2O
II = wall materials	—	1.75	4.95 mg/ml	cryolyzed in 5 ml of H_2O
III = PBS used for washing	100	proportion of dissolved substances 3.05 g	6.56 mg/ml	

Table 2. Results of indirect haemagglutination with sheep sera

	No. of examinations	Negative	Positive (reciprocals of serum dilutions)				
			80	160	320	640	1280
Sheep from infected herd	19	0	0	4	9	3	3
Lambs from infected herd	20	20	0	0	0	0	0
Slaughterhouse sheep	50	47	1	0	2	0	0
Total	89	67	1	4	11	3	3

Table 3. Results of indirect haemagglutination in a heterologous antigen-antibody system

Antiserum	Antigen of *S. gigantea*	Antigen of *S. dispersa*	Antigen of *Toxoplasma*
Sheep anti-*S. gigantea*	1 280	320	160
Mouse anti-*S. dispersa*	160	1 280	0
Man anti-*Toxoplasma*	0	0	2 560
Rabbit anti-*S. cuniculi*	320	640	0
Pig anti-*Sarcocystis* sp.	80	320	0

Sera of surviving mice after experimental infection with *S. dispersa*, serum of a surviving rabbit after experimental infection with *S. cuniculi*, serum of a pig with positive IHA reaction to *S. dispersa* antigen and human serum with a high titre of antibodies in IHA reaction to *Toxoplasma gondii* antigen were used as controls of the course of reaction with heterologous antibodies.

IHA reactions and evaluation of results. The method used was described in detail in the previous communication (Červa and Černá 1982). Optimal values of pH, temperature and time of exposition used for the sensibilization of erythrocytes are identical with the values given for *S. dispersa* antigen.

RESULTS

Already the first attempts to sensibilize the erythrocytes by a standard method showed that the very adsorption of antigen on the surface of the erythrocytes results in their complete agglutination. It was found that the cause of this agglutination is the lectin present in the cryolysates of pure zoites and wall materials of muscle cysts of *S. gigantea*. Identification experiments with lectin, its isolation and biochemical characterization were described in detail in previous papers (Červa et al. 1982, Mácha et al. 1983), in which the former name of the parasite, *S. tenella*, was used.

Inhibition of the effect of lectin. The effect of lectin from *S. gigantea* cysts can be specifically inhibited with 30 mM concentration of D-galactose, N-acetyl-D-galactosamine, methyl-β-D-galactopyranoside, S-allyl-β-D-thiogalactoside and lactose. Lactose is most easily available for the routine serodiagnostics. We have therefore tested the effect of various lactose concentrations and various modifications of IHA reaction in order to neutralize the inhibiting effect of lectin (Červa 1982). The results of our experiments revealed that the bonds between lectin and lactose are unstable and that lectin is released if the sugar concentration decreases. It is therefore necessary to apply minimal lactose concentration of 0.5 % (w./v.) in all solutions used in the reaction, i.e., in Claus—Jensen buffer for washing and diluting of erythrocytes and diluting of examined sera and in Michaelis isotonic buffer used for the dilution of antigen before sensibilization. All these adjusted solutions can be standardly used for IHA with other antigens. The low sugar concentration has no negative effect on the course of reaction and its results may be evaluated after classical criteria.

Specific removal of lectin by means of affinity chromatography. A more laborius way for the removal of lectin from the prepared antigen is affinity chromatography on an adsorbent, where p-amino-phenyl-β-D-galactoside is bound to succinylhydrazido-sepharose (Mácha et al. 1983). The resulting solution can be applied in IHA reaction without any further adjustment.

Efficacy of antigens prepared from various parts of parasitic cysts. All three types of antigens (I—III, see Methods) were titred against a known positive sheep serum. The antigens prepared from a pure suspension of zoites (I) and from cyst walls (II) were effective at the dilutions of 1 : 32 and 1 : 16, which corresponds to the concentration of protein nitrogen of 0.3 mg/ml. Surprisingly effective antigen was the supposed waste, namely PBS used for washing of corpuscular cyst materials (III). The optimal dilution of this antigen in the reaction was 1 : 10 and corresponded to 0.65 mg/ml of protein nitrogen, i.e., to the double of the concentrations of proteins in antigens I and II. However, the erythrocytes sensibilized with this antigen sedimented best of all. The results of haemagglutination given in further text of this paper were obtained with this type of antigen.

Results of IHA with sheep sera. In the group of sheep from the infected herd, 5 of 19 animals did not harbour any *S. gigantea* cysts, 3 sheep harboured only occasional (1—3) cysts in oesophagic muscles. In the remaining animals, numerous cysts were found mainly in oesophagic muscles. Two of them harboured also a high number of

cysts in the muscles of diaphragm. Due to the slaughter organization at the abattoir, the blood samples relevant to the examined organs could not be taken. Consequently, the results of serological examinations cannot be compared with the intensity of infection in muscles of the slaughtered animals. A positive result of IHA reaction was obtained with the sera of all sheep of this group. The distribution of positive findings according to the titre is shown in Table 2. This table also summarizes the results of examination of the other two sets of sheep sera. All lambs from the infected sheep were negative in IHA reaction. Among the sheep bought from individual farmers only 3 animals (6 %) were positive.

Results of IHA in a heterologous antigen-antibody system. The possible relationship could be studied in parasitic antigens from three members of the suborder Eimeriina; in addition to *S. gigantea* also *S. dispersa* and *Toxoplasma gondii* were used. The results are summarized in Table 3. The homologous antibodies evidently prevail and undoubtedly there are common antigenic components not only in *S. gigantea* and *S. dispersa*, but also in *S. cuniculi* from rabbits and *Sarcocystis* sp. infecting pigs in Czechoslovakia. On the other hand, the antigen of *T. gondii* revealed a low antibody titre only in sheep serum. However the specific antibodies to toxoplasmosis, which is quite common in sheep breds, were most probably involved in this case.

Storing of sensibilized erythrocytes. Having regard to practical utilization of IHA reaction in routine diagnostics, it was of interest how long can the sensibilized erythrocytes be stored in a refrigerator at the temperatures of 0—5 °C. It was found that the result of the reaction remains unchanged, without any decrease of the titre, for at least 8 days. The bacterial contamination of erythrocyte suspension, which occurred after a longer storing, was manifested by a non-specific agglutination of erythrocytes, easily distinguishable from the controls.

DISCUSSION

Although the muscle cysts of *S. gigantea*, due to their size, represent an ideal material for the preparation of a pure antigen for serological reactions, this species of sarcosporidians has been only rarely used in serological studies (e.g., Bordjochki et al. 1972, Arru et al. 1978, Boch et al. 1980, Zielasko et al. 1981) and solely in indirect immunofluorescence reaction. Most probably this was due to technical difficulties which arose in classical serological procedures as a result of the presence of a large amount of lectin in the parasitic cysts.

The elimination of lectin effect by a simple process enabled us to try the applicability of indirect haemagglutination for a specific intravital diagnosis of sheep sarcosporidiosis and suitability of *S. gigantea* antigen for the diagnosis of heterologous sarcosporidian antibodies. The results of our studies showed that IHA reaction can be used for both purposes. With regard to the wide distribution of sarcosporidiosis in sheep breds and to the demonstrated harmful effect of this infection on the production, the possibility of intravital diagnostics is of practical importance for the control of this disease.

The antigenically effective substances seem to be evenly distributed in the parasitic cysts. A suitable antigen can be obtained by water extraction of cryolyzed homogenates from whole cysts. The optimal concentration of protein nitrogen according to the titration of antigen corresponds to the values suitable for *S. dispersa* antigen prepared in a similar way (Červa and Černá 1982).

The number of sera which could be obtained is relatively low and cannot demonstrate the occurrence of sarcosporidiosis in sheep breeding in Czechoslovakia. However, some data shown in Table 2 deserve attention. The quantitative distribution

of positive titres in the infected herd suggests a possible relation with the number of parasitic structures in the muscles of the animal. The low number of serologically positive sheep slaughtered at the abattoir corresponds to the absence of macroscopical cysts in the oesophagic muscles. At the same time, the high occurrence of microscopical cysts in these animals indicates that the species *S. gigantea* is antigenically rather distant from other *Sarcocystis* species infecting sheep. The negative findings in 2-3-month-old lambs in the environment where they were exposed to *S. gigantea* infection agree with the known facts on the relatively long period of immunological prepatency (Zielasko et al. 1981, Červa and Černá 1982).

The results of cross reactions with heterologous antisera and antigens may be distorted by concomitant infections common in sheep (*S. ovicanis*, *Toxoplasma gondii*). Only in case of experimentally infected SPF mouse and rabbit and spontaneously infected pig the cross reaction with *S. gigantea* antigen may be supposed. As it has already been reported by other authors, the antigen of *T. gondii* appears to have different structure also according to the results of the IHA test.

The possibility to store for several days the erythrocytes sensibilized with antigen is a very favourable factor for practical utilization of IHA method in extensive serological examinations. The process of sensibilization could be carried out in diagnostical laboratories only once a week, which would considerably extend their capacity.

НЕПРЯМАЯ РЕАКЦИЯ ГЕМАГГЛЮТИНАЦИИ С АНТИГЕНОМ *SARCOCYSTIS GIGANTEA* (RAILLIET, 1886) ASHFORD, 1977

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Резюме. Водный экстракт из криолизированных целых цыст *Sarcocystis gigantea* из мышц овец, несмотря на большое содержание лектина, является подходящим антигеном для определения специфических антител при помощи реакции непрямой гемагглютинации (РНГ). Агглютинирующее действие лектина из цист паразита можно элиминировать с помощью 0,5 % концентрации лактозы, растворенной во всех растворах, применяемых для подготовки РНГ. В сыворотках овец в скотобойне регистрировали положительные титры 1 : 80—1 : 1 280. Положительные реакции в более низких титрах наблюдали также с антителами против *S. dispersa*, *S. cuniculi* и *Sarcocystis* sp. от свиней. Сенсибилизированные эритроциты можно хранить в холодильнике минимум неделю.

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International meeting on the immunology of toxoplasmosis, Lyons

The International meeting on the immunology of toxoplasmosis organized by the Marcel Mérieux Foundation was held in Lyons from 4 to 6 March 1982. The chairmen of this conference were Professor Remington (Palo Alto) and Professor Garin (Lyons). The list of participants included 56 specialists from Austria, Belgium, Czechoslovakia, Denmark, France, Federal Republic of Germany, the Netherlands, Norway and USA. The conference was held in the form of discussion the basic topic being: what was known in 1982 about the immunity to toxoplasmosis in pregnant woman and in infant. The main points of the discussion concerned antigenic structure of toxoplasmas, problems of immunology and physiopathology of toxoplasmosis and humoral and cellular immunity. On the whole three problems were discussed: how to verify that the pregnant woman is protected, how to evaluate seroconversion and how to make sure that the new-born infant is or is not affected.

Different papers dealt with the antigenic structure of *Toxoplasma*, mainly the structure of surface (membrane) antigens, which should be studied in order to develop more sensitive and specific immunoreagents. It was proved that lectins marked with fluorescein are not bound to the surface of tachyzoites. The precipitation properties of surface antigens are variable. Moreover, it was pointed out that the accessory factor of dye test is of complement character. The new method ELIFA (enzyme-linked immuno-filtration-assay) makes it possible to differentiate specifically neonatal antibodies from transplacentally transmitted antibodies. The new method of combining agglutination with ELISA is also specific of the demonstration of IgM antibodies, the so-called IgM-ISAGA (IgM-immuno-sorbent agglutination assay). The method R-EIA (reversed enzyme immunoassay) makes it possible to determine the antibody titre from a single test tube. The method ELISA can be used in studying the circulating immune complexes. The studies on monoclonal antibodies make possible the new procedure of purification and the characterization of antigenic components. The test of lymphoblast transformation specifies the diagnosis of congenital infection. The exoantigens acquired from tissue cultures may be successfully used for skin tests. Also discussed were: the test of basophilic degranulation in toxoplasmic chorioretinitis, the effectiveness of radiated vaccine, the international standard of antitoxoplasma serum, the statewide control of toxoplasma serology in France, the risk of acquiring toxoplasmosis by pregnant woman, the clinical diagnostics of congenital toxoplasmosis and the therapeutic schemes.

The meeting took place in the building of the Institut Mérieux, excellently equipped for conference purposes. The meeting of prominent world specialists in the field of immunology of toxoplasmosis, together with the characteristic French hospitality, contributed to the strengthening of international friendly relations. The conference demonstrated the notable activity of France in this field and the great efforts devoted in the world today to the research of modern diagnostic techniques. However, it was stated that no perfect unity exists so far in the interpretation of the results of diagnostic tests, nor in the prevention of congenital toxoplasmosis. The therapy has not achieved much advancement in this field either.

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